TRANS-SPECIES NUCLEAR TRANSFER

FIELD OF THE INVENTION

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The present invention relates to the field of the cloning of mammals by nuclear transfer, and to a method of producing nuclear transfer embryos from a variety of cell types, including producing embryos using a donor

10 cell from one species and a recipient oocyte from another species. The method may also be used to produce transgenic animals.

BACKGROUND OF THE INVENTION

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Cloning by nuclear transfer was originally described in amphibians (for review see Gurdon, 1986 and DiBerardino, 1987). More recently, cloning by nuclear transfer has been described in the following

20 mammalian species: sheep (Willadsen, 1986; Campbell et al., 1996; and Wilmut et al., 1997); cattle (Robl et al., 1986; Prather et al., 1987; Sims and First, 1993; U.S. Patents 4,994,384, 5,496,720, 5,057,420, 5,453,366); rabbits (Stice and Robl, 1988); pigs

25 (Prather et al., 1989); mice (McGrath and Solter, 1983); and primates (Meng et al., 1997).

Cloning by nuclear transfer in mammals has several common steps. (For review see Prather and First, 1990, incorporated herein by reference.) First, a mature

30 recipient oocyte arrested in Metaphase II is enucleated. Then a donor cell is placed under the zona pellucida of the recipient oocyte and the two are fused together to form a nuclear transfer embryo. Finally, it is necessary to activate the nuclear transfer

35 embryo. Activation is most often accomplished by the same electric pulse which was used to fuse the donor cell and recipient oocyte, but may be also be accomplished by some other stimulus which increases the intracellular calcium concentration of the nuclear

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transfer embryo.

For cows and sheep, it has traditionally been
necessary to use aged recipient oocytes. The reason
for using aged oocytes is that when younger oocytes are
used as recipients the nuclear transfer embryo fails to
activate. Activation is necessary for the new zygote
or nuclear transfer embryo to exit Metaphase II arrest.
While sperm are able to activate bovine oocytes after
24 hours of in vitro maturation, oocytes may be
activated by stimuli which increase intracellular
calcium concentration only after about 42 hours of in
vitro culture. (Susko-Parrish et al., 1994).
Recently, a method has been described by which bovine
oocytes can be successfully activated after only 16
hours of in vitro culture. (Susko-Parish, U.S. Patent
5,496,720, incorporated herein by reference.) This two
step method produces an increase in intracellular
calcium concentration by treatment with the calcium
ionophore inonomycin which is followed by the use of a
serine-threonine kinase inhibitor to suppress cellular
phosphorylation. It is hypothesized that the
serine-threonine kinase inhibitor acts predominantly by
inhibiting the c-Mos protein. c-Mos is thought to be
necessary to maintain Metaphase II arrest, probably by
maintaining the cell cycle protein p34cdc2 in an active
state. p34cdc2 in turn is a protein kinase responsible
for phosphorylating many proteins which are necessary
for oocyte maturation and for normal mitotic cell
division. Fertilization of aged oocytes generally
results in lower rates of development than when younger
oocytes are used. The ability to activate bovine
oocytes at an earlier time after the beginning of in
vitro culture means nuclear transfer embryos can be
activated at a time correlating to the highest rates of
development as determined by in vitro fertilization
studies.

Traditionally, researchers have found that
differentiated cells could not be used for nuclear

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transfer because they would fail to support complete
development. Studies in amphibians indicated that as
more and more developmentally advanced nuclei are used
for nuclear transfer, development decreases. (For
5   review see Gurdon, 1986.) For this reason, many
researchers concentrated on culturing embryonic stem
cells (ES cells). ES cells are derived from
undifferentiated germ cells or embryos and are cultured
so as to maintain the cells in an undifferentiated,
10   pluripotential state. ES cells were first described
for the mouse and are used to create chimeric embryos
since nuclear transfer does not work in mice. (For
review see Stewart, 1991; Anderson, 1992.) Nuclear
transfer embryos have been produced from ES cells in
15   cattle (Sims and First, 1993) and sheep (Campbell et
al., 1996, incorporated herein by reference). However,
these methods are somewhat inefficient since only low
numbers of the ES cells can be produced.

Recently, researchers have described a method for
20   the production of nuclear transfer sheep embryos from a
differentiated adult cell line and from cell lines
derived from differentiated embryos (Wilmut et al.,
1997, incorporated herein by reference). The donor
cells are serum starved by culturing in low serum
25   medium for five days before nuclear transfer. This
causes the cells to arrest in G_0 The researchers
hypothesize that nuclei arrested in G_0 are more
amenable to nuclear remodeling and reprogramming.
Nuclear remodeling and reprogramming describe the
30   events whereby the donor cell nucleus is altered
morphologically and biochemically to resemble a normal
early embryo nucleus in morphology and transcriptional
competence. (For review see Prather and First, 1990;
Fulka et al., 1996.) In effect, the donor cell nucleus
35   is de-differentiated.

This new nuclear transfer procedure may have
several important impacts. First, genetically
identical clones may be made of adult animals with

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economically valuable traits such as high milk
production. Second, the large numbers of donor cells
which may be generated by normal cell culture
techniques make possible the use of standard cell
transfection techniques. For example, the donor cells
may be transfected with exogenous DNA and a selectable
marker. (For review see Joyner, 1991; Stewart, 1991.)
Additionally, homologous recombination techniques can
be used to add or delete genes from the donor cells at
specific sites within the genome. (Koller et al.,
1989; Cappecchi, 1989; Stanton et al., 1990, herein
incorporated by reference.) After genetic modification
of the donor cells by transfection with a selectable
marker or homologous recombination, the donor cell can
be used in the nuclear transfer procedure to produce a
transgenic animal. The availability of these
techniques should greatly increase the efficiency of
producing transgenic animals other than mice. These
animals may be made transgenic for economically
valuable traits or for pharmaceutical proteins which
may be harvested from the animals milk or blood.
Third, this new technique of nuclear transfer may be
used to propagate endangered species where it would be
impossible to obtain enough gametes to optimize cloning
procedures.

SUMMARY OF THE INVENTION

It is an object of the present invention to
provide an efficient method of producing cloned embryos
from differentiated cells by nuclear transfer. It is
another object of this invention to provide a method
for cloning donor cells of one species by utilizing a
recipient oocyte from another species. It is still
another object of this invention to provide cloned
embryos. The cloned embryos may be implanted into
recipient animals to produce offspring or used to
originate totipotent or pluripotent cell lines for

therapeutic uses.   It is also an object of the present
invention to provide methods for efficient production
of transgenic animals and animals in which genes have
been "knocked out" by homologous recombination.

5   Therefore, the present invention provides a method
of producing nuclear transfer bovine embryos from
bovine somatic donor cells and bovine recipient oocytes
or from somatic donor cells of one species and
recipient oocytes of another species. The donor cells

10   may be selected from the group consisting of embryonic
derived cells, germ cells, somatic cells, and
genetically modified cells.   In this method, the donor
cells are isolated and then cultured in low serum
medium to induce the donor cells to undergo G_0 arrest.

15   The donor cells are then fused to an enucleated
recipient oocyte to create a nuclear transfer embryo.
When enucleated bovine recipient oocytes are used, the
oocytes are preferably selected from the group of
bovine oocytes undergoing nuclear maturation within 16

20   hours of beginning in vitro culture. Fusion may
preferably be accomplished by application of an
electric pulse 16-24 hours after the beginning of in
vitro culture.   The nuclear transfer embryo must then
be activated.   Preferably, activation is accomplished

25   by elevating intracellular calcium within the embryo by
the use of ionomycin and then incubating the embryo
with a serine threonine kinase inhibitor such as
dimethylaminopurine (DMAP).   The activation step is
preferably performed about from 4 to 16 hours after

30   fusion, and most preferably from about 4 to 8 hours
after fusion. Alternatively, activation may be
performed about 16 to 52 hours after the beginning of
in vitro culture, most preferably about 20 to 32 hours
after the beginning of in vitro culture.

35   The present invention also provides embryos
produced by the above process and totipotent or
pluripotent cell lines derived from those embryos.
These embryos individually comprise either cytoplasm

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derived from a bovine oocyte, bovine cytoplasm derived
from a differentiated bovine cell, cell membrane
derived from a bovine oocyte, cell membrane derived
from a differentiated bovine cell and nuclei derived

5   from a differentiated bovine cell or cytoplasm and cell
membrane from one species and differentiated cytoplasm,
differentiated cell membrane, and nuclei derived from a
differentiated cell of another species.

10   DESCRIPTION OF THE PREFERRED EMBODIMENT

Cloning mammals by nuclear transfer is generally
comprised of the following steps. First, the recipient
oocyte is enucleated. Second, the donor cell is placed

15   in contact with the enucleated oocyte and the two are
fused together to form a nuclear transfer embryo.
Third, the nuclear transfer embryo is activated so that
development can occur. The basic cloning steps for
bovine embryos have been described in U.S. Patents

20   4,994,384, 5,496,720, 5,057,420, and 5,453,366,
incorporated herein by reference. Cloning procedures
for sheep have been described by Willadsen, 1986,
Campbell et al., 1996, and Wilmut at al., 1997,
incorporated herein by reference.

25   Recently, researchers in Scotland demonstrated
that somatic cells and embryonically derived cells
could be used as donor cells for nuclear transfer.
(Wilmut et al., 1997; Campbell et al., 1996.) The
subject matter of these publications also appears in

30   PCT Publications WO 97/07669, WO 97/07668 and WO
96/07732, incorporated herein by reference. Prior to
these results, it was thought impossible to clone
mammals or fully developmentally competent amphibians
by nuclear transfer from cells which have undergone

35   differentiation. (DiBerardino, 1997.) The entire
concept of the use of embryonic stem cells to produce
embryos was based on keeping the stem cells in
undifferentiated state. The use of stem cells for

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cloning purposes is described in PCT Publications WO
95/17500, WO 95/16770 and WO 95/08625, herein
incorporated by reference.

The use of somatic cells or other differentiated
cells for nuclear transfer in mammals has many
potential uses. First, the cells of a genetically
valuable animal such as a cow with high milk production
may be used to make genetically identical cows.
Second, endangered species could be propagated. Third,
transgenic animals could be produced from cell lines
transfected with exogenous DNA. The high numbers of
somatic cells available mean that well developed means
of transfecting cells with exogenous DNA can be used
such as the use of selectable markers to select
transfected cells and homologous recombination. By
using these methods researchers should be able to
produce transgenic animals far more efficiently than by
currently used techniques such as pronuclear injection.

One of the most important aspects of this
invention is the wide variety of cells which may be
used. The term "donor cell", as used herein, means any
cell of any species which is used to provide a nucleus
to an enucleated oocyte. Types of donor cells include,
but are not limited to, somatic cells which are cells
derived from adult tissue, embryonic derived cells
which are differentiated cells harvested from any stage
of embryo or fetus, germ cells which are cells which
form or a product of the germline lineage, and
genetically modified cells which are cells into which
exogenous DNA has been introduced by any transfection
technique.

The donor cells used in the present invention must
be serum starved before use. (Wilmut et al., 1997;
Campbell et al., 1996.) Serum starvation induces the
donor cells to arrest in the G_0 stage of the cell
cycle. Serum starvation is a simple technique whereby
the normal concentration of serum in cell culture
media, generally about 10 percent, is substantially

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lowered for several days. For example, Wilmut et al.,
cultured the donor cells used in their experiments for
5 days in a medium with 0.5 percent fetal calf serum.
The reason why this treatment produces cells which may
5   be used as donor cells for nuclear transfer is unknown.
One theory is that the serum starvation causes changes
in chromatin structure which allows for reprogramming
of the donor nucleus by the recipient oocyte. An
additional benefit of serum starvation is that the
10   cells uniformly arrest at a defined stage of the cell
cycle, G_0. G_0 is a stage of quiescence in cell growth
the cells enter into just after mitosis. Actively
growing cells generally enter into G1 and prepare for
DNA synthesis if not serum starved. By inducing the G_0
15   arrest, all the cells used for subsequent cloning are
at the same stage of the cell cycle, in contrast to
actively growing cells which are at various stages of
the cell cycle. The advantage is that the G_0 arrested
cells have a 2N amount of DNA which corresponds to the
20   normal amount of DNA contributed to a newly fertilized
egg by the oocyte and sperm. Therefore, the nuclear
transfer embryos begin development with the correct
amount of DNA and correct ploidy.

The term "low serum medium" as used herein means
25   cell culture medium that is supplemented with a lower
than normal amount of serum, such as fetal calf serum,
so that cells cultured in the medium undergo G_0 arrest.
"G_0 arrest" means that stage of the cell cycle after
mitosis and before DNA synthesis where the cell is
30   quiescent and ceases preparation for DNA synthesis and
further cell division. The medium used may be any
medium in which the donor cell type is normally
cultured. Examples of such media include but are not
limited to Ham's F 10, TCM-199, and DMEM. In the
35   preferred embodiment of this invention, donor cells are
cultured in low serum medium for a period of time
sufficient to induce G_0 arrest. In the most preferred
embodiment, the donor cells are cultured for five days

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in alpha-MEM with no serum.

The next step in cloning by nuclear transfer involves the culture, selection and enucleation of the recipient oocyte. "Recipient oocyte" means a mature oocyte which is at the correct stage of development to be fertilized by a sperm cell and which after enucleation, serves as the recipient for the donor cell nucleus. For cloning procedures in most mammals, it is necessary to use mature oocytes which are arrested in Metaphase II. (Prather and First, 1990.) This is the stage at which fertilization leading to competent development takes place in most mammalian species. For species such as dogs in which fertilization takes place at a different stage of oocyte development, it would be necessary to utilize an oocyte of the appropriate stage. The use of Metaphase II oocytes also facilitates enucleation because the Metaphase II plate is located adjacent to the first polar body.

The recipient oocyte may be matured either in vitro or in vivo. In the preferred embodiment of the invention, the recipient oocyte is a mature oocyte of any species. In the most preferred embodiment, the recipient oocyte is a mature bovine oocyte. In vivo matured bovine oocytes may be recovered surgically from either superovulated or nonsuperovulated cows or heifers 35 to 48 hours past the onset of estrus or past an injection of human Chorionic Gonadotropin (hCG) or similar hormone. In vitro matured bovine oocytes may be prepared by the methods of Sirard et al., (1988) Parrish et al., (1988) and Rosenkrans and First (1993), incorporated herein by reference. Briefly, ovaries are collected at a slaughterhouse and transported to the laboratory in normal saline at 30 to 34° C. Transport varies from 2 to 6 hours. Oocytes from small follicles of 1 to 6 mm in diameter are aspirated. The immature oocytes are then washed three times in TL HEPES without glucose and to which 0.22 mM pyruvate and 1 mg/ml BSA have been added. The immature oocytes are then placed

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in 50 μl drops of maturation medium under paraffin oil.
Maturation medium is composed of TC199
containing 10 percent heat treated fetal calf serum,
0.22 mM pyruvate, 0.5 μg/ml FSH-P (Scherring-Plough
5   Animal Health Corp. Kenilworth, N.J.) and 1 μg/ml
estradiol. Ten oocytes are placed in each drop and
incubated overnight at 39° C, in high humidity
atmosphere comprised of 5% CO_2 and 95% air. The term
"beginning of in vitro culture" means the time at which
10   the recipient oocytes are placed in maturation medium.

In a further preferred embodiment, the recipient
bovine oocytes are selected from the group of oocytes
which mature within 16 hours of the beginning of in
vitro culture. To select such oocytes at 16 hours, it
15   is necessary to remove the enclosing cumulus cells from
the oocytes so that the extrusion of the first polar
body may be observed. Cumulus may be removed by
placing the oocytes in TL-HEPES medium supplemented
with 2.0 mg/ml of hyaluronidase and then gently
20   aspirating the oocytes with a fire-polished pipette
tip. (Sims and First, 1994.) Oocytes which have
extruded the first polar body are in Metaphase II
arrest and mature. The reason for selecting this
subpopulation of oocytes is that they support a higher
25   level of embryo development after fertilization as
demonstrated by Dominko and First, 1997.

In the preferred embodiment, recipient oocytes are
enucleated at an appropriate time after maturation has
occurred, normally 1 to 15 hours after extrusion of the
30   first polar body. In the most preferred embodiment,
bovine oocytes are enucleated 16-20 hours after the
beginning of in vitro culture. The procedure for
enucleation is as follows and is described in Prather
et al., U.S. Patent 4,994,384, incorporated herein by
35   reference. Cumulus cells are removed from the oocytes
by placing the oocytes in TL-HEPES medium supplemented
with 2.0 mg/ml of hyaluronidase and then gently
aspirating the oocytes with a fire-polished pipette

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tip. (Sims and First, 1994.) Oocytes to be enucleated
are then placed in culture dishes in 100 μl drops under
oil of TL-HEPES medium supplemented with calcium and
magnesium. (See generally Sims and First, 1994.)

5   Micromanipulation may be performed with a Nikon Diaphot
microscope equipped Hoffman optics and Narishige
micromanipulators. The recipient oocyte is held with a
holding pipet with a 120 μm outer diameter and 30 μm
inner diameter. Enucleation is performed by using a

10   beveled, sharpened enucleation pipet with an outer
diameter of approximately 25 μm. The inner diameter
may be varied according to the size of the donor cell
which is used. To enucleate the oocyte, the
enucleation pipet is inserted into the oocyte and a

15   small amount of cytoplasm is removed just below the
first polar body. Preferably, enucleation can be
verified by staining the mature recipient oocyte with
Hoechst 33342, visualizing DNA by excitation emission
upon exposure to ultraviolet light for a short time

20   (i.e. 5 seconds), enucleating the recipient oocyte, and
washing the oocyte 2 times in TL-HEPES.

        The next steps involve placing the donor cell
under the zona pellucida of the recipient oocyte and
fusing the recipient oocyte with donor cell. The donor

25   cell is placed under the zona pellucidae of the
recipient oocyte by use of the enucleation pipet. In
the preferred embodiment the donor cell and the
recipient oocyte may come from the same species or
different species and are fused at the time appropriate

30   for the recipient oocyte species. Examples include but
are not limited to a bovine somatic cell with a bovine
recipient oocyte, a sheep somatic cell with a bovine
recipient oocyte, or a genetically altered or unaltered
non-human primate cell or human cell with a bovine

35   oocyte.

        In the most preferred embodiment, a donor cell
which has been cultured in low serum medium is fused at
16-24 hours after initiation of in vitro culture to a

recipient bovine oocyte selected from the group of
oocytes maturing within 16 hours from the beginning of
culture. Two main advantages exist for utilizing this
method of cloning for bovine donor cells and bovine

5   recipient oocytes. First, genetically valuable cows,
such as those that exhibit high milk production or
other valuable traits, can be multiplied from the
various types of donor cells. Second, transgenic
animals can be produced in an efficient manner.

10   Standard methods of DNA transfection along with
selectable markers can be utilized with large
populations of donor cells. (For review see Joyner,
1991; Stewart, 1991.) Also, homologous recombination
techniques can be used to introduce exogenous DNA or to

15   knock out endogenous genes. (Koller et al., 1989;
Cappecchi, 1989; Stanton et al., 1990.) The exogenous
DNA introduced into the donor cells may be a gene
encoding a valuable trait or a gene encoding a
pharmaceutical protein which will be harvested from the

20   animal.

These advantages are also applicable when the
donor cells and recipient oocytes are derived from
different species. In another most preferred
embodiment, donor cells from species including but not

25   limited to sheep, pigs, rats, non-human primates and
humans are fused to the bovine recipient oocyte. An
additional advantage of this method is that nuclear
transfer clones of these species may be made utilizing
the well-defined bovine recipient oocyte. Maturation

30   procedures and timing for fusion and activation have
been optimized in the bovine system. When bovine eggs
are used, these two parameters do not have to be
optimized as they would need to be if a recipient
oocyte from another species is utilized. This may be

35   of special importance in the cloning of endangered
species where there are too few animals from which
gametes can be obtained to conduct experiments to
optimize maturation, culture conditions, and timing for

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developmental competence.

Using the bovine oocyte as the recipient oocyte
for donor cells of different species raises several
interesting questions. Nuclear transfer utilizing
5   donor cells and recipient oocytes from different
species provides a powerful experimental system to
analyze early embryo development and the common
characteristics of early embryo development shared
across species. First, it is known that embryos of
10   different species undergo a period of maternally
directed cleavage divisions. These cleavage divisions
occur in the absence of transcription from the
embryonic genome. The time at which the embryo begins
transcription is termed the maternal to embryonic
15   transition (MET). (For review see Telford et al.,
1990.) For example, bovine embryos begin transcription
at the late 4 to early 8 cell stage, murine embryos at
the 2 cell stage, rat embryos at the 2 cell stage,
sheep embryos at the 8 cell to 16 cell stage and
20   primate embryos at the 4 cell stage. (Telford et al.,
1990.) So the timing of MET is specific to each
species. Likewise, the complement of proteins which is
initially produced by the embryonic genome seems to
vary among species where this has been studied.
25   (Crosby et al., 1988; Barnes and First, 1991; Connover
et al., 1991.)

Here, the inventors describe the surprising result
that the bovine recipient oocyte is capable of
supporting the MET of various species when donor cells
30   of various species are fused to the bovine recipient
oocyte to produce a nuclear transfer embryo. Factors
present in the bovine recipient oocyte cytoplasm are
therefore probably responsible for the MET and
subsequent transcription and translation of the species
35   characteristic MET proteins because the pre-MET embryo
is transcriptionally incompetent. This is evidenced by
the fact that the nuclear transfer embryos progress
through the MET and that the first readily observable

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signs of differentiation, compaction and blastocoel
formation, occur as in normal embryos.

Furthermore, the bovine recipient oocyte is
apparently capable of supporting nuclear remodeling in
the donor cells of various species. Nuclear remodeling
refers to the series of changes that occur to the
nucleus of the donor cell after nuclear transfer. (For
review see Fulka et al., 1996; Prather and First,
1990.) The morphological and biochemical changes in
the nucleus are probably due to factors present in the
cytoplasm of the recipient oocyte. These changes
include the disappearance of differentiated nucleoli,
swelling of the transferred nucleus, and in amphibians
the exchange of proteins between the nucleus and
cytoplasm. These changes are probably necessary to
support further development. Again, the bovine
recipient oocyte apparently has the necessary proteins
to support this remodeling in other species.

Third, the bovine recipient oocyte is probably
capable of supporting correct changes in methylation of
the DNA of the donor cells of the various species. DNA
methylation has been shown to be a mechanism for the
regulation of gene expression in mammals. (For review
see Brandeis et al., 1993.) The DNA of mouse gametes
is heavily methylated. Upon fertilization the DNA of
the newly formed zygote is demethylated. At
gastrulation, a bimodal pattern of methylation is
formed with CpG island containing genes being
demethylated and most other genes being methylated.
Tissue specific genes are demethylated as
differentiation continues. The bovine recipient oocyte
may be able to support the initial wave of
demethylation as evidenced by the developmental
competence of the nuclear transfer embryos. After the
MET, the genome of the donor cell probably exerts
itself to establish correct methylation patterns.

In the preferred embodiment, fusion of the
recipient oocyte and donor nucleus is performed by

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electric pulse. In the most preferred embodiment, the bovine recipient oocyte is fused to the bovine donor cells or donor cells of other species by a double electric pulse in sucrose or sorbitol fusion medium in a Zimmerman Electrofusion Instrument (CGA Corporation, Chicago, Illinois). The method is similar to that described in Prather et al., U.S. Patent 4,994,384. The Electrofusion Instrument may be adjusted as follows:

       Fusion voltage: 90 volts (DC, 1.8 KV/cm);
       Electrode distance: 0.5 millimeter;
       Alignment voltage: 1-5 volts (AC);
       Alignment frequency: 1000 KHZ;
       Pulse duration: 30 microseconds, two pulses;
       Postfusion alignment time: 5 seconds

Sorbitol fusion medium consists of 0.25 M d-sorbitol in medical grade H20 (Baxter), 100 μM Ca-acetate, 0.5 mM Mg-acetate, and 1 g/l Fatty Acid Free BSA (pH 7.2 at room temperature, 255 mOsm). Sucrose fusion medium consists of 95.84 g/l sucrose, 0.107 g/L Mg-acetate-4-H_2O, 0.016 , μM Ca-acetate, 0.174 g/l KH_2PO_4 anhydrous, 0.031 g/l glutathione, and 0.01 g/l Fatty Acid Free BSA, ELISA grade (260-290 mOsm, pH 7.0). Other fusion media may be used such as Zimmerman Cell Fusion Medium, mannitol, and phosphate buffer solution.

       Alternatively, fusion may be accomplished by treatment of the donor cell and recipient oocyte with polyethylene glycol as described in Sims and First, 1994. Fusion may be accomplished also by use of Sendai virus. (Graham, 1969.)

       The next step in cloning by nuclear transfer is activation of the nuclear transfer embryo. "Nuclear transfer embryo" means the embryo which results from the fusion of the donor cell and recipient oocyte. Normally, the oocyte is activated by the sperm when fertilized. The interaction of the sperm with the

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oocyte cell membrane causes an elevation of
intracellular calcium which starts a cascade of events
including the release of the new zygote from Metaphase
II arrest. Activation by fertilization is followed by
pronuclear formation. Since nuclear transfer embryos
are not fertilized, an activation stimulus is needed to
release the nuclear transfer embryo from Metaphase II
arrest. In traditional nuclear transfer protocols, the
electric pulse used to fuse the donor cell and
recipient oocyte acts as the activation stimulus.
(Willadsen, 1986; Robl et al., 1986; Prather et al.,
1987; Campbell et al., 1996.) It is necessary that
sheep recipient oocytes and bovine recipient oocytes be
aged in order for an electric pulse to be sufficient to
activate the resulting nuclear transfer embryo.
(Prather et al., 1987; Campbell et al., 1996.)

In a preferred embodiment, the nuclear transfer
embryo is activated by a stimulus sufficient to release
the nuclear transfer embryo from Metaphase II arrest.
"Activate", as used herein, means the use of a stimulus
sufficient to release the nuclear transfer embryo from
Metaphase II arrest. In an alternative embodiment, a
bovine nuclear transfer embryo is activated by the
fusion electric pulse described above at 35 to 52 hours
after the beginning of in vitro culture.

Recently, a method has been described for
activating bovine oocytes as early as 10 hours after
the beginning of in vitro culture. (Susko-Parish et
al., U.S. Patent 5,496,720, incorporated herein by
reference.) This method comprises the sequential
incubation of an oocyte or nuclear transfer embryo with
a calcium ionophore such as ionomycin, and a
serine-threonine kinase inhibitor such as 6-
dimethylaminopurine (DMAP). Treatment with ionomycin
causes an increase in intracellular calcium similar to
that normally caused by the sperm at fertilization.
The role of DMAP is less clear, but it may act by
inhibiting the serine-threonine kinase c-Mos which is

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thought to stabilize the activity of p34cdc2 which is
activated by phosphorylation on serine and threonine
residues and inactivated by phosphorylation on tyrosine
residues. High p34cdc2 activity is generally

5   associated with Metaphase II arrest. Since the
activity of c-Mos is inhibited, p34cdc2 activity is
down regulated via the absence of an entity able to
maintain the activating phosphorylations on p34cdc2's
serine and threonine residues. One reason that aged

10   oocytes may be activated solely by electrical pulse is
that p34cdc2 activity in these cells has declined. All
that is needed to activate aged oocytes is the
elevation of intracellular calcium by one of several
methods. To overcome the high levels of p34cdc2

15   activity in younger oocytes, both elevation of
intracellular calcium and inhibition of
serine-threonine kinases appear to be required.

In the preferred embodiment, nuclear transfer
embryos are activated at an appropriate time

20   (preferably about 4 to 16 hours after fusion) by
increasing the concentration of intracellular calcium
and incubation with a serine-threonine kinase
inhibitor. In the most preferred embodiment, nuclear
transfer embryos comprised of fused bovine recipient

25   oocytes and bovine donor cells or donor cells from
other species are activated by sequential incubation
with ionomycin and DMAP at about 28 hours after the
beginning of in vitro culture. Briefly, the nuclear
transfer embryos are exposed to 5 μM ionomycin (5 mM

30   stock in DMSO; Calbiochem, La Jolla, CA) in TL-HEPES (1
mg/ml fatty acid free BSA) for 4 minutes. The nuclear
transfer embryos are then diluted into TL-HEPES (30
mg/ml fatty acid free BSA) for five minutes and then
further diluted into TL-HEPES (1 mg/ml Fraction V BSA).

35   The nuclear transfer embryos are then moved to 50 μl
drops under paraffin oil of CRlaa with 1.9 mM DMAP and
incubated for 4 hours at 39° C, 5% CO_2 in air, high
humidity. After this incubation, embryos are diluted

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in TL HEPES with 1 mg/ml BSA and then transferred into
CRlaa media and cultured at 39° C, 5% CO_2 in air, high
humidity.   Other methods which may be used to increase
intracellular calcium include ethanol treatment,
A23187, electric shock and caged chelators.   Other
serine-threonine kinase inhibitors which may be used
include staurosporine, 2-aminopurine, and sphingosine.
For a further description of these methods see
Susko-Parrish et al., U.S. Patent 5,496,720,
incorporated herein by reference.

     There are two main advantages to the use of the
ionomycin/DMAP activation regimen.   First, the nuclear
transfer embryo may be activated at the time when
developmental competence is highest as demonstrated by
studies of the fertilization of bovine oocytes.
Traditional cloning techniques require that the
recipient oocyte be aged before fusion and activation.
The use of aged oocytes may compromise the development
of the resulting nuclear transfer embryo.   Second,
p34cdc2 has been shown to have a positive effect on
nuclear remodeling.   (Fulka et al., 1996.)   The nuclei
of nuclear transfer embryos which are exposed to a
cellular environment with high levels of p34cdc2
activity remodel more completely and more quickly than
when fusion occurs to recipients that have already been
preactivated or which have low levels of p34cdc2
activity.   One reason that aged oocytes may be
activated solely by electrical pulse is that p34cdc2
activity in these cells has declined.   Since p34cdc2
activity has declined in aged recipient oocytes,
nuclear remodeling may not be complete.   With the
ionomycin/DMAP regimen, at the time of fusion, which
occurs prior to activation, the donor cell nucleus is
exposed to high levels of p34cdc2 activity which is
beneficial to nuclear remodeling.

     Temporal separation of the fusion and activation
steps allows the nuclear transfer embryo sufficient
time to remodel its nucleus.   In the most preferred

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embodiment, fusion occurs at 16 hours after the
beginning of in vitro culture while activation occurs
28 hours after the beginning of in vitro culture. This
procedure differs from previously described nuclear

5   transfer techniques where fusion and activation are
simultaneous. The examples verify that this method
results in an approximately 2 fold increase to
blastocyst than previously described somatic cell
cloning methods.

10   After activation of the nuclear transfer embryo,
it is necessary to either culture the embryo in vitro
or transfer the embryo to an appropriate recipient
animal. In the preferred embodiment, nuclear transfer
embryos comprised of bovine recipient oocytes and

15   bovine donor cells are cultured until the blastocyst
stage in vitro and then transferred to a recipient. In
vitro culture is carried out as described in Sims and
First, 1994 and Rosenkrans et al., 1993. Briefly,
nuclear transfer embryos may be cultured in a defined

20   medium, CR1aa or CR2 for 7-8 days at 39° C in 5%
CO_2/95% air in high humidity. CR1aa is comprised of 5
mM hemicalcium lactate, 114.7 mM sodium chloride, 3.1
mM potassium chloride, 26.2 mM sodium bicarbonate, and
3 mg/ml fatty acid free bovine serum albumin.

25   (Rosenkrans et al., 1993.)

                        EXAMPLES

Methods Common to all Experiments

30

Oocyte maturation- In vitro matured bovine oocytes may
be prepared by the methods of Sirard et al. (1988),
Parrish et al. (1988) and Rosenkrans and First (1993).
Briefly, ovaries are collected at a slaughterhouse and

35   transported to the laboratory in normal saline at 30°
to 34° C. Transport time varies from 2 to 6 hours.
Oocytes from small follicles of 1 to 6 mm in diameter
are aspirated. The immature oocytes are then washed

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three times in TL-HEPES without glucose and to which
0.22 mM pyruvate and 1 mg/ml BSA have been added. The
immature oocytes are then placed in 50 μl drops of
maturation medium under paraffin oil. Maturation
5   medium is composed of TC199 containing 10 percent heat
treated fetal calf serum, 0.22 mM pyruvate, 0.5 μg/ml
FSH-P (Scherring-Plough Animal Health Corp. Kenilworth,
N.J.) and 1 μg/ml estradiol. Ten oocytes are placed in
each drop and incubated overnight at 39° C, in high
10   humidity atmosphere comprised of 5% CO_2 and 95% air.

Micromanipulation- Enucleation was performed at 16-20
hours after the beginning of in vitro culture. The
procedure for enucleation is as follows and is
15   described in Prather et al., U.S. Patent 4,994,384,
incorporated herein by reference. Cumulus cells are
removed from the oocytes by placing the oocytes in
TL-HEPES medium supplemented with 2.0 milligrams per
milliliter of hyaluronidase and then gently aspirating
20   the oocytes with a fire-polished pipette tip. (Sims
and First, 1994.) Oocytes to be enucleated are then
placed in culture dishes in 100 microliter drops under
oil of TL-HEPES medium supplemented with calcium and
magnesium. (See generally Sims and First, 1994.)
25   Micromanipulation may be performed with a Nikon Diaphot
microscope equipped Hoffman optics and Narishige
micromanipulators. The recipient oocyte is held with a
holding pipet with a 120 μm outer diameter and 30 μm
inner diameter. Enucleation is performed by using a
30   beveled, sharpened enucleation pipet with an outer
diameter of approximately 25 μm. The inner diameter
may be varied according to the size of the donor cell
which is used. To enucleate the oocyte, the
enucleation pipet is inserted into the oocyte and a
35   small amount of cytoplasm is removed just below the
first polar body. Preferably, enucleation can be
verified by staining the mature recipient oocyte with
Hoechst 33342, visualizing DNA by excitation emission

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upon exposure to ultraviolet light for a short time
(i.e. 5 seconds), enucleating the recipient oocyte, and
washing the oocyte 2 times in TL-HEPES. The donor cell
is placed under the zona pellucidae of the recipient
5   oocyte by use of the enucleation pipet.

Fusion- Fusion of the bovine, non-human primate, rat,
sheep and porcine donor cells to the bovine recipient
oocytes was performed at 16-24 hours after the
10   beginning of in vitro culture. The bovine recipient
oocyte is fused to the bovine donor cells or donor
cells of other species by a double electric Zimmerman
Electrofusion Instrument (CGA Corporation, Chicago,
Illinois). The method is similar to that described in
15   Prather et al., U.S. Patent 4,994,384. The
Electrofusion Instrument was adjusted as follows:

         Fusion voltage: 90 volts (DC, 1.8 KV/cm);
         Electrode distance: 0.5 millimeter;
20   Alignment voltage: 1-5 volts (AC);
         Alignment frequency: 1000 KHZ;
         Pulse duration: 30 μseconds, two pulses;
         Postfusion alignment time: 5 seconds

25   Two types of fusion media were utilized. The first
consisted of 0.25 M d-sorbitol in medical grade H_2O
(Baxter), 100 μM Ca-acetate, 0.5 mM Mg-acetate, and 1
g/1 Fatty Acid Free BSA (pH 7.2 at room temperature,
255 mOsm). The second consisted of 95.84 g/1 sucrose,
30   0.107 g/L Mg-acetate-4-H_2O, 0.016 μM Ca-acetate, 0.174
gA KH_2PO_4 anhydrous, 0.031 g/1 glutathione, and 0.01
g/1 Fatty Acid Free BSA, ELISA grade (260-290 mOsm, pH
7.0).

35   Cell culture- Somatic cells were obtained from bovine,
sheep, non-human primate (Rhesus), porcine and rat ear
skin samples, removed from live animals. The skin
samples were placed into PBS (phosphate buffered

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saline, pH) supplemented with antibiotic/antimycotic
(Gibco), and washed 3 times. All hair was manually
removed and the sample cut into small pieces. The cut
samples were then placed in 0.05% trypsin/1 mM EDTA
5   solution (Gibco) for 40 minutes at 30° C and stirred
frequently during incubation. The samples were then
centrifuged (10 minutes, 1000 rpm) and supernatant
containing single cells removed to remove undigested
pieces of tissue. The final supernatant was placed
10   into alpha-MEM (Gibco), supplemented 10% fetal calf
serum (HiClone) and antibiotic/antimycotic (Gibco) and
cultured in tissue flasks (Falcon, 25 cm^2) at 37° C for
at least a week. After a monolayer of cells had
formed, the medium was removed and the monolayer washed
15   with Ca-Mg free PBS once and replaced by trypsin/EDTA
solution and incubated at 37° C for 2-3 minutes. Cells
are then desegregated by pipetting and centrifuged as
described above. The pellet of cells was diluted 1:2
with alpha-MEM and placed into two flasks. All
20   subsequent passages were performed in the same manner
every 2 days thereafter.

For nuclear transfer, the cells were removed from
flasks as described for passaging and placed into a 35
mm Petri dish (Falcon tissue culture) and grown in
25   alpha-MEM for 2-12 days in the absence of any serum.
On the day of nuclear transfer, the cells grown in the
absence of sera were trypsinized as described above,
washed in Ca-Mg free PBS and placed into TL HEPES
supplemented with 0.1 -3 mg/ml BSA Faction V, 0.2 mM
30   Na-pyruvate and 25 µg/ml gentamicin.

Activation of nuclear transfer embryos- Activation was
performed at 24-28 hours after the beginning of in
vitro culture. The nuclear transfer embryos were
35   exposed to 5 µM ionomycin (5 mM stock in DMSO;
Calbiochem, La Jolla, CA) in TL-HEPES (1 mg/ml fatty
acid free BSA) for 4 minutes. The nuclear transfer
embryos were then diluted into TL-HEPES (30 mg/ml fatty

acid free BSA) for five minutes and then twice diluted
into TL-HEPES (3 mg/ml Fraction V BSA). The nuclear
transfer embryos were then moved to 50 microliter drops
under paraffin oil of CR1aa supplemented with 1.9 mM

5   DMAP and incubated for 4 hours at 39° C, 5% CO_2 in air,
high humidity.

Culture after activation- After removal from DMAP,
nuclear transfer embryos were diluted twice in TL HEPES

10   supplemented with 3 mg/ml BSA Fraction V. All embryos,
regardless of species, were cultured in CR1aa. CR1aa
is comprised of 5 mM hemicalcium lactate, 114.7 mM
sodium chloride, 3.1 mM potassium chloride, 26.2 mM
sodium bicarbonate, and 3 mg/ml fatty acid free bovine

15   serum albumin. (Rosenkrans et al., 1993.) Incubations
were conducted at 39° C, 5% CO_2 in air, high humidity.
After three days of culture, 10% heat treated FCS was
added to all embryos, regardless of species and the
embryos allowed to develop to blastocyst.

20

Results

Nuclear transfer embryos were constructed from
bovine recipient oocytes and the following cell lines:

25   bovine adult fibroblasts (BAF), bovine fetal
fibroblasts (BFF), sheep adult fibroblasts (SAF),
porcine adult fibroblasts (PAF), non-human primate
adult fibroblasts (MAF) and rat adult fibroblasts
(RAF). The results are summarized in Table 1.

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Table 1: Ability of donor cells to initiate embryonic development after nuclear transfer into a recipient bovine oocyte.

Donor Cell Line	Nuclear Transfer (N)	Fused (N)	Lysed or not fused(N)	CLV.* (%/fused)	CM+BL** (%/C.V.)
BAF	220	145	75	81/145	22/81
BFF	82	51	31	31/51	18/31
SAF	327	182	135	120/135	38/120
PAF	93	36	57	32/57	12/32
RAF	108	33	75	37/	na
MAF	30	28	2	24/28	14/24

* CLV. - number of embryos undergoing cleavage

** CM-BL- number of embryos developing to compact morula or blastocyst

Column 1 indicates the donor cell line, column 2, Nuclear Transfer, indicates the number of nuclear transfers attempted, column 3, Fused, indicates the number of fused nuclear transfer embryos produced, column 4, Lysed, indicates the number of recipient oocytes which Lysed or where fusion did not occur, column 5, CLV, indicates the number nuclear transfer embryos which cleaved and is expressed as a percentage of fused nuclear transfer embryos, column 6, CM+BL, indicates the number of nuclear transfer embryos developing to compacted morula or blastocyst and is expressed as a percentage of cleaved embryos.

These results indicate the bovine oocyte can be utilized as the recipient oocyte in combination differentiated donor cells from cattle, pigs, monkeys, rats and sheep to create nuclear transfer embryos. The methods employed lead to a greater efficiency of development to compacted morula and blastocyst than previously described somatic cell cloning procedures. Wilmut et al., reported the development of 29 embryos

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to compacted morula or blastocyst out of 277 fused
nuclear transfer embryos (11.7%) when using somatic
sheep cells.   Here the researchers report the
development of 38 compacted morulae or blastocysts out
5   of 182 fused nuclear transfer embryos (20.9%),   An
increase in efficiency of about two fold.
        To date, 13 embryos derived from bovine adult
fibroblasts have been transferred to recipient cows, 8
embryos derived from sheep adult fibroblasts have been
10   transferred to sheep, and 20 embryos derived from rat
adult fibroblasts have been transferred to rats.

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